Genomic imprinting is a method of gene regulation whereby a gene is expressed in a parent-of-origin-dependent fashion; however, it is hypothesized that imprinting should not occur in oviparous taxa such as birds. Therefore, we examined the allelic expression of two genes in the chicken that are reciprocally imprinted in most mammals, mannose 6-phosphate/insulin-like growth factor 2 receptor (M6P/IGF2R) and insulin-like growth factor 2 (IGF2). Single nucleotide polymorphisms were identified in these genes, and cDNA was prepared from several tissues of embryos heterozygous for these polymorphisms. Both alleles of M6P/IGF2R and IGF2 were expressed in all tissues examined by RT-PCR. Since the expression of these genes was independent of the parent from which they were inherited, we conclude that neither M6P/IGF2R nor IGF2 are imprinted in the chicken.

**Keywords** Genomic imprinting · Biallelic expression · Chicken · IGF2 · M6P/IGF2R

**Introduction**

Genomic imprinting is a phenomenon whereby gene expression is parent-of-origin dependent. It appears to have evolved over 100 million years ago (Killian et al. 2000) and the actively debated genetic conflict hypothesis purports that it resulted from a parental genetic “tug-of-war” to control maternal-dependent growth of mammalian offspring (Moore and Haig 1991). According to this hypothesis, growth of the offspring should be promoted by paternally expressed genes and limited by those that are maternally expressed. Furthermore, the duration and extent of prenatal growth and postnatal care should be major driving forces in the evolution of genomic imprinting.

The reciprocal imprinting of mannose 6-phosphate/insulin-like growth factor 2 receptor (M6P/IGF2R) and insulin-like growth factor 2 (IGF2) in mice and the phenotypes of mice deficient in their expression provide support for the genetic conflict hypothesis. Paternally expressed IGF2 encodes for a critical fetal mitogen, and mice deficient in this growth factor have a dwarf phenotype (DeChiara et al. 1990) while mice over-expressing Igf2 are larger than normal (Leighton et al. 1995). The M6P/IGF2R in viviparous mammals binds IGF2 and phosphomannosyl glycoproteins through independent binding sites and limits the biological activity of IGF2 by targeting the growth factor for degradation in lysosomes (Nolan et al. 1990; Kornfeld 1992; Dahms et al. 1993; Killian et al. 2000). The M6PR/IGF2R in mice is expressed only from the maternal allele (Barlow et al. 1991), and animals lacking M6PR/IGF2R accumulate excess IGF2, have somatic overgrowth and die perinatally (Lau et al. 1994; Wang et al. 1994; Ludwig et al. 1996; Nolan and Lawlor 1999).

The genetic conflict hypothesis also predicts that genes that regulate embryonic development would not be imprinted in oviparous taxa because in these animals genes expressed during embryogenesis cannot influence the amount of resources they receive from the mother (Haig and Graham 1991). However, recent work in the chicken examining the imprinted status of IGF2 has yielded conflicting results. Koski et al. (2000) reported that IGF2 was expressed monoallelically in some embryos from either paternal or maternal alleles. At the same time O’Neill et al. (2000) reported that IGF2 was expressed in chick embryos in a strictly biallelic manner. Therefore, we re-examined the allelic expression of chicken IGF2 and examined expression of chicken M6PR/IGF2R for the first...
time, and discovered that expression was biallelic for both genes. Our findings are consistent with the predictions of the genetic conflict hypothesis.

Materials and methods

Fertile chicken eggs (White Leghorn or Barred Plymouth Rock strains) were generated at North Carolina State University Department of Poultry Science by artificial insemination of females and were incubated at 37.5°C and 60% relative humidity. Three-day-old embryos were dissected free of membranes, frozen immediately on dry ice and stored at –80°C. Individual tissues (i.e., liver, lungs, heart, skeletal muscle and brain) were removed from 17-day-old embryos and treated similarly. Blood was withdrawn from adult chickens into heparinized syringes and frozen at –80°C.

DNA and total RNA were isolated from whole adult blood or from embryos or tissues using DNA-STAT 120 or RNA-STAT-60 according to the manufacturer's instructions (Tel-test, Friendswood, Tex.). RNA (1–5 µg) was reverse transcribed using Superscript II and oligodT primers, according to the manufacturer's instructions (Life Technologies, Grand Island, N.Y.), and 1/40 of the cDNA obtained was used as a template for PCR amplification. PCR was routinely performed in a 30-µl reaction volume using 1.5 U platinum Taq DNA polymerase (Life Technologies, Baltimore, Md.), 15 pmol primers, 1.5 mM MgCl₂, and 100 µM dNTPs (94°C × 15 s, 65°C × 5 s, and 72°C × 60 s for 35 cycles). Amplification products were purified from agarose gels using GenElute spin columns (Sigma, St Louis, Mo.). They were sequenced manually using radiolabeled terminator cycle sequencing kit (USB Corporation, Cleveland, Ohio) or with an automated ABI 377 sequencer (PE Biosystems, Foster City, Calif.) using the manufacturer’s BigDye terminator cycle sequencing kit.

Polymorphic sites were identified in the chicken M6PR/IGF2R using genomic DNA amplified with primers designed to putative exon 48, M6PR48F2 (5′-gggaaggagaa-agatgtcatggtggtcc-3′) and M6PR48R1 (5′-ggaatacccacgtcatatcttttattg-aaattgc); the amplified fragment was sequenced using the ABI 377 sequencer (PE Biosystems, Foster City, Calif.). For analysis of allelic expression of the M6PR/IGF2R, cDNA was amplified using M6PR48R1 and a primer located in exon 47 of the gene, M6PR47F1 (5′-ctgcaagagacatgatggctggtcc-3′); the amplified fragment was sequenced using M6PR48R1. Chicken M6PR/IGF2R intron 2 was amplified using primers Int2F (5′-gttggagaatactgacatgggtcc-3′) and Int2R (5′-gttgtctctctgtttttaaatttggc-3′), designed with reference to sequences in exon 2 and exon 3, respectively (Zhou et al. 1995). The Expand Long Template PCR system (Roche Boehringer Mannheim, Indianapolis, Ind.) was used with buffer 3 and amplification conditions of 94°C ×10 s, 61°C ×20 s, and 68°C ×4 min for 30 cycles. The amplified fragment was sequenced using intron-specific primers (sequences available from the authors by request) by a combination of manual and automatic sequencing, and was analyzed by Grail informatics (http://compbio.ornl.gov/grail-bin/EmptyGrailForm). The complete sequence of intron 2 of the chicken M6P/IGF2R is deposited in GenBank (Accession No. AF305581).

To identify single nucleotide polymorphisms in chicken IGF2, a portion of the gene was amplified using primers IGF2F2 (5′-ccctccagccccctcaactaggagaa-agatgtcatggtggtcc-3′) and IGF2R2 (5′-tccctccagccccctcaactagagctctgt-3′) and sequenced using the ABI 377 sequencer. These primers span an intron and were also used to amplify IGF2 cDNA. Allelic expression was analyzed by manual sequencing with the primer IGF2R5 (5′-ccctccagccccctcaactaggagaa-3′).

Results and discussion

Imprint status of IGF2 in chickens

IGF2 has been highly conserved in vertebrate evolution and is imprinted in all investigated eutherian and marsupial mammals with only the paternal allele being expressed (DeChiara et al. 1990; Rainier et al. 1993; Overall et al. 1997; Feil et al. 1998; O’Neill et al. 2000). Tissue-specific biallelic expression of IGF2 does occur, however, in mouse and human brain (Hu et al. 1995; Pham et al. 1998) and adult human liver (Vu and Hoffman 1994). Allelic expression of IGF2 has also been analyzed in the chicken, with independent studies generating conflicting results (Koski et al. 2000; O’Neill et al. 2000). Further analysis of IGF2 expression in this species is therefore required.

We identified a T to C transition in exon 3 of the IGF2 coding sequence of White Leghorn chickens (position 936 of Accession no. AF218827; position 943 of Accession no. S82962). Amplified genomic DNA prepared from skeletal muscle of 17-day-old chicken embryos (n=18) identified six individuals heterozygous for this single nucleotide polymorphism (SNP). RNA and cDNA were then prepared from liver, lungs, heart, skeletal muscle and brain of these informative embryos. Both IGF2 alleles were found to be expressed in all tissues examined (Fig. 1). We also found biallelic IGF2 expression in heterozygous 3-day-old embryos (n=17, data not shown). These results are consistent with the conclusion of O’Neill et al. (2000) that IGF2 is not imprinted in the chicken and agree with the prediction of the genetic conflict hypothesis.

![Fig. 1 Allelic expression of the chicken insulin-like growth factor 2 (IGF2). Sequence analysis of genomic DNA amplified from skeletal muscle of a 17-day-old chicken embryo identifies an individual heterozygous for a T to C polymorphism (arrow, sequenced in reverse direction). Similar analysis of cDNA prepared from embryonic tissues of the same embryo demonstrates expression of both alleles. The analysis shown is representative of all informative embryos analyzed.](image-url)
Conflict hypothesis that genes that regulate embryonic development would not be imprinted in members of oviparous taxa (Moore and Haig 1991).

Koski et al. (2000) also examined IGF2 expression in chicken embryos and reported that 33% of chickens have monoallelic expression from either the paternal or maternal allele. They concluded that imprinting at the IGF2 locus is a polymorphic trait. Thus, there is a significant discrepancy between these results and our observations and those of O’Neill et al. (2000). This discrepancy does not result from IGF2 imprinting being dependent upon stage of development since we found biallelic IGF2 expression in 3-day-old embryos, the same age studied by Koski et al. (2000). Each of the heterozygote embryos we examined was based on separate individual matings of an out-bred line of chickens and we did not find any evidence for polymorphic IGF2 imprinting. There are several possible reasons for the observed difference in imprint status of chicken IGF2. Apparent monoallelic expression of a non-imprinted IGF2 gene could result from the preferential PCR amplification of a single allele when template DNA/cDNA concentration is low. It could also result from genetic anomalies such as somatic mosaicism in the parents or loss of heterozygosity at the IGF2 locus in embryos. Such anomalies do occur in the chicken and karyotypic mosaicism (Abdel-Hameed and Shoffner 1971) and triploidy (Ohno et al. 1963) can be compatible with viable postnatal life in this species. In addition parthenogenetic chickens can develop to maturity (Sarvela 1973). Rather than inferring the genotype of embryos from those of their parents, embryos should be accurately genotyped to guarantee that they are heterozygous at the investigated polymorphic locus. It may be significant that the embryos analyzed by Koski et al. (2000) were not reported as having been genotyped and that monoallelic expression was limited to the offspring of one specific male. Therefore, the random monoallelic expression described by Koski et al (2000) clearly does not constitute the parent-of-origin-dependent expression that is the hallmark of imprinted genes. Taken together with the results of the current study, the available evidence is most consistent with IGF2 not being imprinted in chickens.

Imprint status of M6P/IGF2R in chickens

In viviparous mammals the M6P/IGF2R encodes for a receptor that binds both phosphomannosyl glycoproteins and IGF2 through independent binding sites (Kornfeld 1992; Dahms et al. 1993; Yandell et al. 1999). The platypus, chicken and Xenopus receptors lack the ability to bind IGF2, however (Clairmont and Czech 1989; Zhou et al. 1995; Killian et al. 2000). It has been proposed that IGF2 binding by M6P/IGF2R evolved in response to high levels of paternally produced IGF2 (Haig and Graham 1991), and that natural selection would have favored inactivating the paternal allele once the receptor acquired its ability to bind IGF2. According to this hypothesis, M6P/IGF2R is predicted to be biallelically expressed in the chicken as it is in monotremes (Killian et al. 2000).

Allelic expression of the chicken M6P/IGF2R was determined with a G to A transition SNP at nucleotide position 8,637 in the 3’UTR (Accession No. U35037). Embryos heterozygous for the polymorphism were identified by sequencing of genomic DNA isolated from the skeletal muscle of 24 embryos. RNA was then extracted from several tissues of these informative embryos (5 White Leghorn, and 2 Barred Plymouth Rock). RT-PCR was used to amplify the region containing the polymorphism, and the amplified fragment was sequenced to identify expressed alleles. In all heterozygous embryos examined and in all tissues examined (i.e., liver, lungs, heart, skeletal muscle and brain) both alleles of M6P/IGF2R were expressed (Fig. 2). We also showed biallelic expression of M6P/IGF2R in blood of heterozygous adult chickens (n=21, data not shown). Therefore, we conclude that the chicken M6P/IGF2R is not imprinted. Thus, the M6P/IGF2R’s acquisition of IGF2 binding, and its resultant ability to limit embryonic growth by degrading IGF2, appears to be closely related to the evolution of imprinting at the M6P/IGF2R locus.

The molecular mechanism by which the imprinted status of the M6P/IGF2R is achieved and maintained...
is currently unclear. Monoallelic expression of the M6P/IGF2R in mice is purported to depend on differential methylation of a CpG island in the second intron (region 2; Stöger et al. 1993). Hybridization studies initially indicated that region 2 was present in primates, mice and birds (Stöger et al. 1993). Although a homologous element was reported in humans (Smrzka et al. 1995), the human M6P/IGF2R is expressed from both alleles (Kalscheuer et al. 1993; Ogawa et al. 1993). The relationship of mouse region 2 to that of the human is unclear, as there is no significant sequence homology between them. Nevertheless, they share a high level of organizational similarity with human region 2, also in intron 2, containing a CpG island with maternal-specific methylation. In contrast to initial indications that region 2 was widely conserved (Stöger et al. 1993; Smrzka et al. 1995), the platypus and opossum M6P/IGF2R do not have a region 2-like element (Killian et al. 2000). Since the opossum M6P/IGF2R is expressed only from the maternally inherited allele, either the mechanism of M6P/IGF2R imprinting in the opossum differs from that of the mouse, or region 2 may not be the primary regulator of imprinting at this locus.

To test for the presence of an avian element with features similar to those of mouse and human region 2, we amplified and sequenced the entire second intron of the chicken M6P/IGF2R. The intron was approximately 5 kb in length, smaller than those of mouse, human and platypus, but similar to that of the opossum (Fig. 3; Stöger et al. 1993; Smrzka et al. 1995; Killian et al. 2000). A chicken repeat 1 (CR1) element was found at the 3′ end of the intron (Vandergon and Reitman 1994). However, the intron had no features characteristic of a CpG island. It did not have a high G+C content or any clustering of CpG dinucleotides (Fig. 3), and G/C boxes, a regular occurrence in CpG islands (Gardiner-Garden and Frommer 1987), are entirely missing from the sequence. Thus, although region 2 was reported to be present in birds (Stöger et al. 1993), our results demonstrate that chicken M6P/IGF2R intron 2 does not contain a CpG island analogous to those in mice and humans (Stöger et al. 1993; Smrzka et al. 1995). The absence of region 2 in the chicken, platypus and opossum, together with the lack of sequence homology between mouse region 2 and human region 2, suggest that such elements may have evolved independently in several mammalian orders. Analysis of the M6P/IGF2R in additional marsupial and eutherian mammals is necessary to clarify the involvement of region 2 in regulating imprinting of the M6P/IGF2R.

In conclusion, our demonstration that chicken IGF2 and M6P/IGF2R are both expressed biallelically is consistent with the predictions of the genetic conflict hypothesis of imprint evolution (Moore and Haig 1991). Our data also support the idea that the reciprocal imprinting of these genes in viviparous mammals is closely related to their opposing effects on embryonic growth.

Acknowledgements We would like to acknowledge the expert technical assistance of Michael Kulik and the expertise of the Duke DNA sequencing facility and thank the members of the Jirtle laboratory for critical reading of the manuscript. Laboratory animal technician support of the NCSU Scott Hall poultry facility is greatly appreciated. This study was supported by NIH grants CA25951 and ES08823 and DOD grant DAMD17-98-1-8305.


References


